Journal of Industrial Microbiology (1995) 15, 407–410

Dependence of nitrogen- and phosphorus-regulation of β-lactam antibiotic production by *Streptomyces clavuligerus* on aeration level

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Interference with β-lactam production in *Streptomyces clavuligerus* by ammonium and phosphate ions, normally observed with optimum levels of aeration, was eliminated by restriction of the air supply. Under such a restriction, ammonium slightly stimulated and phosphate markedly stimulated β-lactam antibiotic production. These are rare examples of 'regulation reversal' by an environmental modification.

Keywords: nitrogen regulation; phosphorus regulation; cephamycin; secondary metabolism; antibiotics; *Streptomyces clavuligerus*

Introduction

When ammonium or phosphate ions are provided to *Streptomyces clavuligerus* at levels satisfying the needs of N or P for maximum growth, secondary metabolism, ie production of β-lactam antibiotics, is suppressed. This interference occurs under otherwise optimum conditions for antibiotic production which include high levels of aeration. In the present study, we show that such negative regulatory effects are dependent on high aeration. At low levels of aeration, ammonium and phosphate no longer interfere and can actually stimulate secondary metabolism.

Materials and methods

Microorganisms

Streptomyces clavuligerus NRRL 3585 (ATCC 27064) is a Gram-positive filamentous soil bacterium. This actinomycete produces a variety of β-lactam antibiotics including penicillins, cephalosporins (including cephamycin C) and clavulanic acid. Its main product is cephamycin C. Spores were produced on a sporulation agar containing (g L^{-1}): yeast extract 4; malt extract 10; glucose 4; agar 20; pH was adjusted to 7.3. After harvesting, the spores were stored at −20° C in 20% (w/v) glycerol.

Escherichia coli ESS was the assay organism for β-lactam antibiotics. It is a mutant of *E. coli* W which is supersensitive to β-lactam antibiotics.

Fermentation

The seed medium was trypticase-soy broth (TSB, Difco Laboratories, Detroit, MI, USA), 30 g L^{-1}, adjusted to pH 7.0. The chemically-defined fermentation medium contained (g L^{-1}): glycerol 10; L-asparagine 2.0 (15 mM); L-lysine 1.83 (10 mM); MOPS 21 (100 mM); $MgSO_4 \cdot 7H_2O$ 0.6; K_2HPO_4 3.5 (20 mM) and trace salts solution (containing $FeSO_4 \cdot 7H_2O$ 1.0; $MnCl_2 \cdot 4H_2O$ 1.0; $ZnSO_4 \cdot H_2O$ 1.0 and $CaCl_2$ 1.0), 1 ml L^{-1}. The pH was adjusted to 6.8 with KOH.

Incubation conditions

All liquid medium incubations were done in unbaffled 500-ml Erlenmeyer flasks at 30° C on a rotary shaker (2″ diameter). The normal fermentation was done at 200 rpm whereas oxygen-limited fermentations were done at 120 rpm.

Fifty milliliters of seed medium per flask were inoculated with 1 ml spore suspension and incubated for 38–40 h. Normal flask fermentations were conducted in 50 ml media, inoculated with 2.5 ml seed and incubated for at least 8 days. Oxygen-limited flask fermentations were conducted in 350 ml media, inoculated with 17.5 ml seed, and incubated for at least 8 days.

Assays

Growth was estimated by absorbance using a Klett Summerson colorimeter (Klett Manufacturing Co, New York, NY, USA) with a red filter. For the measurements, 0.5 ml or 1.0 ml of whole broth was added to an equal volume of 2.5 N HCl. Water was added to 10 ml and the suspension treated in the Branson Sonifier (Branson Ultrasonics Co, Danbury, CT, USA) for 30 s. The absorption of the sonicated suspension was determined in the range of 50–150 Klett units. The Klett units were multiplied by the dilution factor. Under these conditions, a dry cell weight (DCW) of 1 g L^{-1} is equivalent to 270 units.

β-Lactam antibiotics were determined with the agar plate-disk diffusion assay with the assay strain seeded in LB agar. Cephalosporin C was used as standard and *E. coli* ESS as assay organism. Repeated assays showed a maximum variation of 12%.

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Received 6 February 1995; accepted 4 June 1995

Results

Limitation of growth by decreasing aeration

To study the effect of aeration limitation on regulation, we limited aeration by increasing medium volume from the optimum volume of 50 ml per 500-ml unbaffled Erlenmeyer flask shaken at 120 rpm. Growth decreased in both rate and extent by increasing volume from 50 ml to 400 ml (Figure 1). For further experiments, a volume of 350 ml was chosen.

Effect of phosphate

Increased potassium phosphate was added to the chemically-defined fermentation medium under optimum aeration (50 ml per flask, 200 rpm; Figure 2) and limited aeration (350 ml per flask, 120 rpm; Figure 3) conditions. It can be seen from Figure 2 that, as expected, increased phosphate suppressed β-lactam production under optimum aeration while stimulating the extent of growth. On the other hand, under limited aeration as shown in Figure 3, increased phosphate exerted a reversal of regulation, ie it stimulated β-lactam production while growth was virtually unaffected. In the experiments described in Figures 2 and 3, the pH remained between 6.6 and 7.1

Effect of ammonium

A similar experiment was done with ammonium. NH_4Cl (120 mM) was added to chemically-defined medium under the two aeration conditions. Figure 4 shows the expected suppression of β-lactam production under optimum aeration; no effect was observed on growth. Under limited aeration (Figure 5), NH_4^+ showed a slight stimulatory effect on β-lactam production without affecting growth. In the fermentations described in Figures 4 and 5, the pH remained within 6.6 and 7.0.

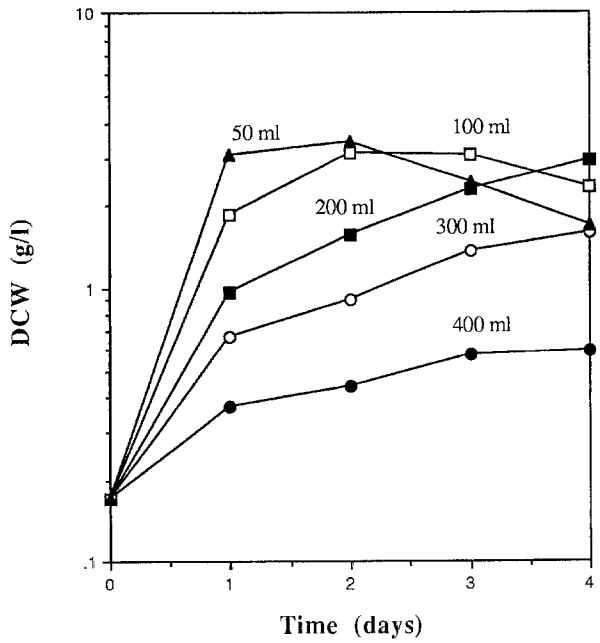


Figure 1 Effect of increased medium volume per 500-ml flask on growth

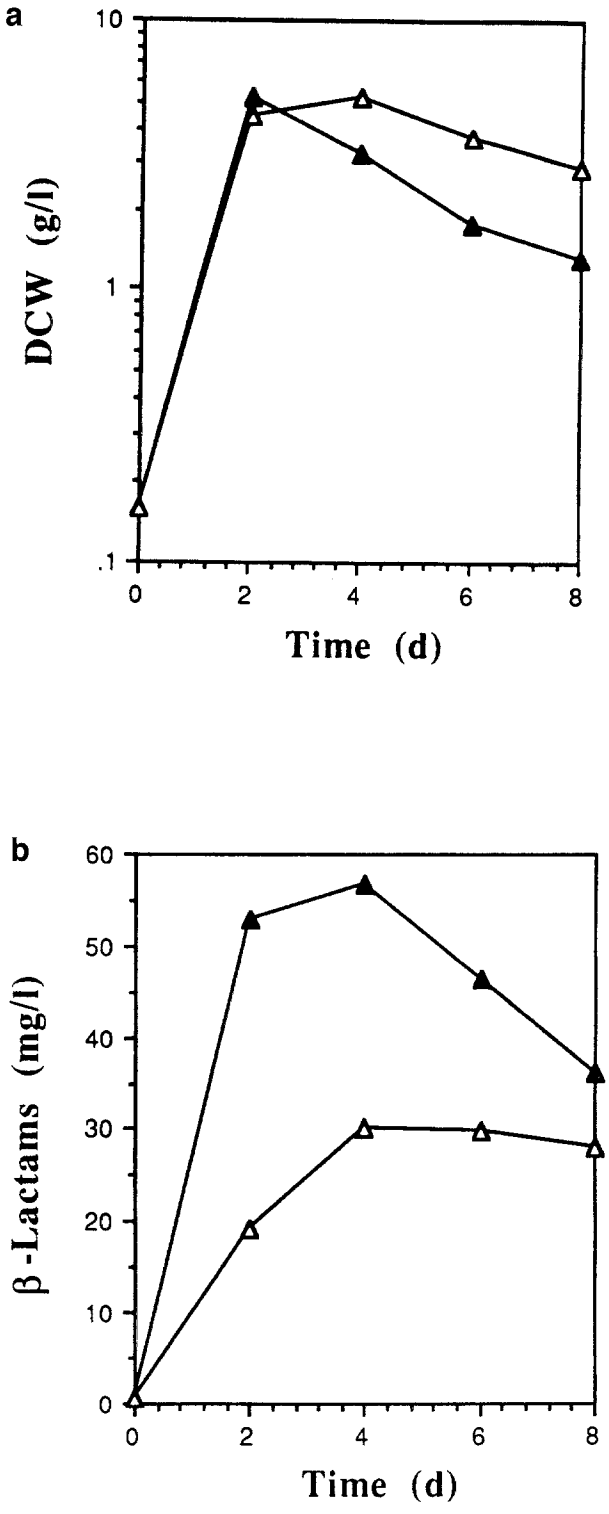
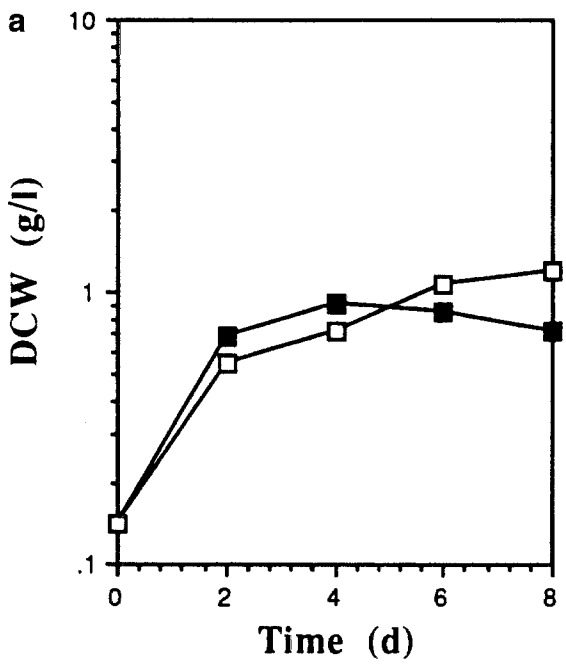


Figure 2 Effect of increased phosphate concentration (100 mM) on growth (a) and β-lactam production (b) under optimum aeration conditions. ▲, normal phosphate; △, increased phosphate

Discussion

The negative effect of phosphate on β-lactam production in *S. clavuligerus* was first noted by Aharonowitz and Demain [1] and confirmed by Lubbe *et al* [7], Lebrihi *et al* [6] and Zhang *et al* [10]. Its mechanism involves



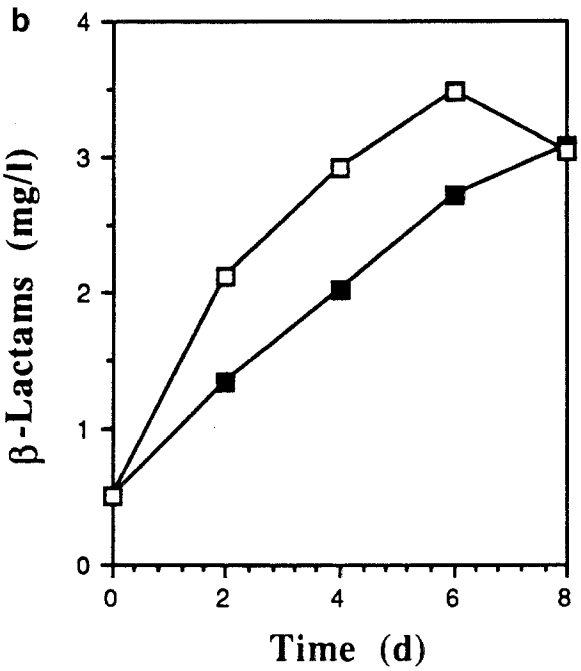
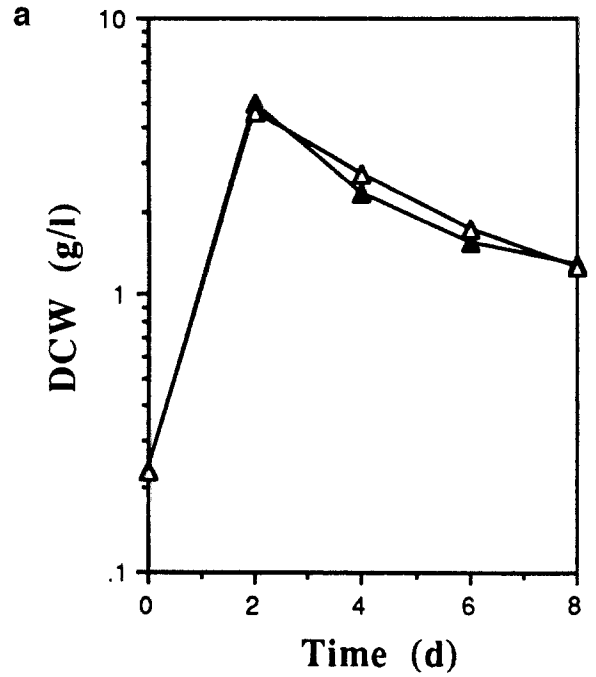


Figure 3 Effect of increased phosphate concentration (100 mM) on growth (a) and β-lactam production (b) under poor aeration conditions. ■, normal phosphate; □, increased phosphate



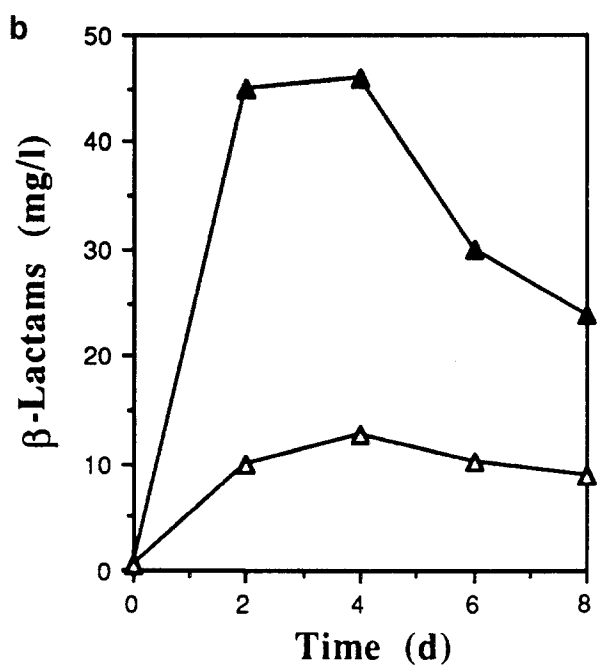


Figure 4 Effect of ammonium chloride (120 mM) on growth (a) and β-lactam production (b) under optimum aeration conditions. ▲, no ammonium chloride; △, ammonium chloride added

repression of the biosynthetic enzymes δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (cyclase), penicillin epimerase and deacetoxy-cephalosporin C synthase (expandase) and inhibition of ACVS and cyclase. The suppression of β-lactam production by ammonium salts in *S. clavuligerus* was observed by Aharonowitz and Demain [2] and confirmed by Brana *et al* [4] and Zhang *et al* [11]. The mechanism involved is repression of ACVS, cyclase and expandase as well as induction of alanine dehydrogenase and elevation of the intracellular level of alanine which can inhibit the activity of these three enzymes [5].

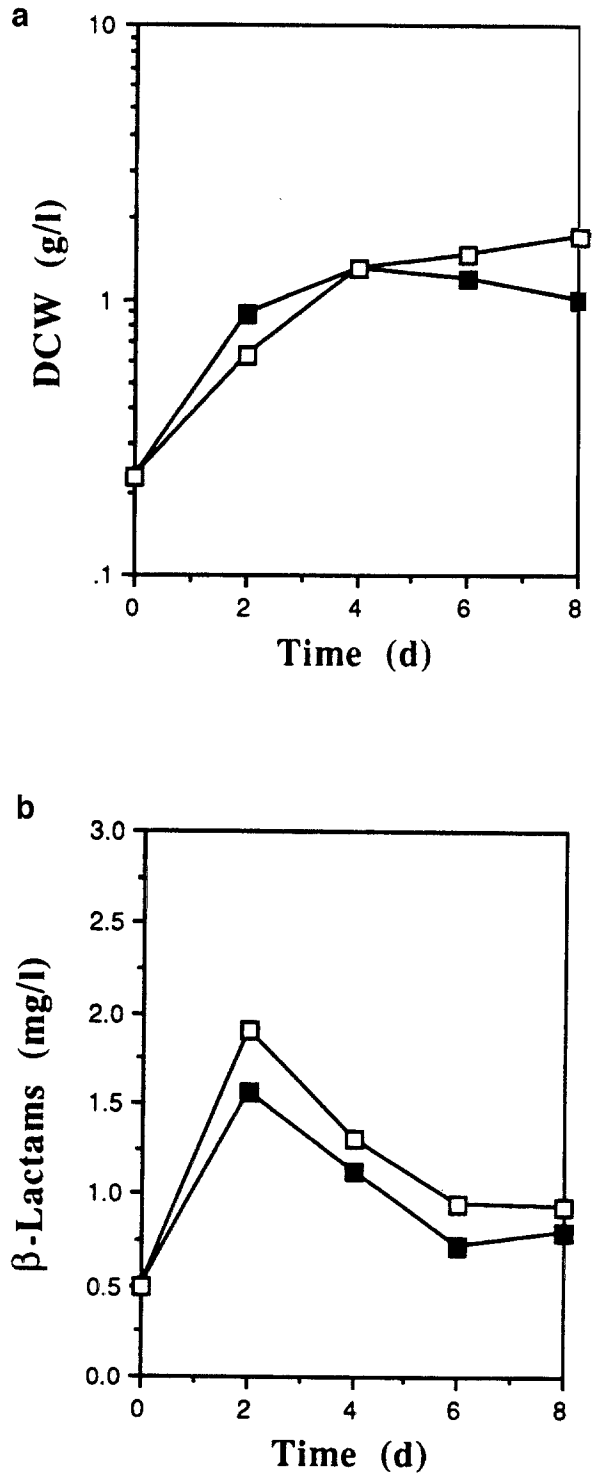


Figure 5 Effect of ammonium chloride (120 mM) on growth (a) and β-lactam production (b) under poor aeration conditions. ■, no ammonium chloride; □, ammonium chloride added. In this experiment, fresh medium was added at each sampling. This was done in an attempt to keep aeration limited throughout the entire fermentation.

These negative effects occur under optimum fermentation conditions which include high levels of oxygen [8,9]. In this work, we found that the regulatory controls are eliminated and even reversed by limitation of aeration. The removal of these controls is not due to changes in growth. The relationship between nutrient regulation and oxygen level is unknown but we previously reported that carbon source regulation by glycerol in resting cells of *S. clavuligerus* requires oxygen limitation [3], an effect opposite to those observed here with nitrogen and phosphorous regulation.

Acknowledgements

We are indebted to NASA for grant no NAG 9-602. We thank Duane L Pierson, Saroj K Mishra and David W Koenig for encouragement.

References

1 Aharonowitz Y and AL Demain. 1977. Influence of inorganic phosphate and organic buffers on cephalosporin production by *Streptomyces clavuligerus*. Arch Microbiol 115: 169–173.
2 Aharonowitz Y and AL Demain. 1979. Nitrogen nutrition and regulation of cephalosporin production in *Streptomyces clavuligerus*. Can J Microbiol 25: 61–67.
3 Brana AF, W-S Hu and AL Demain. 1983. Involvement of aeration in carbon source regulation of cephem antibiotic biosynthesis in *Streptomyces clavuligerus*. Biotechnol Lett 5: 791–794.
4 Brana AF, S Wolfe and AL Demain. 1985. Ammonium repression of cephalosporin production by *Streptomyces clavuligerus*. Can J Microbiol 31: 736–743.
5 Kasarenini S and AL Demain. 1994. A role for alanine in the ammonium regulation of cephalosporin biosynthesis in *Streptomyces clavuligerus*. J Ind Microbiol 13: 217–219.
6 Lebrihi A, P Germain and G Lefebvre. 1987. Phosphate repression of cephamycin and clavulanic acid production by *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 26: 130–135.
7 Lubbe C, S Wolfe and AL Demain. 1985. Repression and inhibition of cephalosporin synthetases in *Streptomyces clavuligerus* by inorganic phosphate. Arch Microbiol 140: 317–320.
8 Rollins MJ, SE Jensen, S Wolfe and DWS Westlake. 1990. Oxygen derepresses deacetoxycephalosporin C synthase and increases the conversion of penicillin N to cephalosporin C in *Streptomyces clavuligerus*. Enzyme Microb Technol 12: 40–45.
9 Yegneswaran PK, MR Gray and BG Thompson. 1991. Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. Biotechnol Prog 7: 246–250.
10 Zhang J, S Wolfe and AL Demain. 1989. Phosphate regulation of ACV synthetase and cephalosporin biosynthesis in *Streptomyces clavuligerus*. FEMS Microbiol Lett 48: 145–150.
11 Zhang J, S Wolfe and AL Demain. 1989. Ammonium ions repress δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase in *Streptomyces clavuligerus* NRRL 3585. Can J Microbiol 35: 399–402.